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Thermoinactivation Mechanism of Glucose Isomerase

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Abstract

In this article, the mechanisms of thermoinactivation of glucose isomerase (GI) from *Streptomyces rubiginosus* (in soluble and immobilized forms) were investigated, particularly the contributions of thiol oxidation of the enzyme's cysteine residue and a "Maillard-like" reaction between the enzyme and sugars in high fructose corn syrup (HFCS). Soluble GI (SGI) was successfully immobilized on silica gel (13.5 μm particle size), with an activity yield between 20 and 40%. The immobilized GI (IGI) has high enzyme retention on the support during the glucose isomerization process. In batch reactors, SGI (half-life = 145 h) was more stable than IGI (half-life = 27 h) at 60°C in HFCS, whereas at 80°C, IGI (half-life = 12 h) was more stable than SGI (half-life = 5.2 h). IGI was subject to thiol oxidation at 60°C, which contributed to the enzyme's deactivation. IGI was subject to thiol oxidation at 80°C, but this did not contribute to the deactivation of the enzyme. SGI did not undergo thiol oxidation at 60°C, but at 80°C SGI underwent severe precipitation and thiol oxidation, which caused the enzyme to deactivate. Experimental results show that immobilization suppresses the destabilizing effect of thiol oxidation on GI. A "Maillard-like" reaction between SGI and the sugars also caused SGI thermoinactivation at 60, 70, and 80°C, but had minimal effect on IGI. At 60 and 80°C, IGI had higher thermostability in continuous reactors than in batch reactors, possibly because of reduced contact with deleterious compounds in HFCS.

Index Entries: Deactivation; immobilized enzyme; kinetics; silica gel; thermostability; glucose isomerase.

Introduction and Background

High fructose corn syrup (HFCS), produced enzymatically using immobilized glucose isomerase (GI), dominates 70% of today's nutritive sweetener market. Because of poor enzyme thermostability and byproduct formation, the current commercial glucose isomerization reaction can only be carried out at 60°C, producing ≤ 50% fructose (55% fructose is more desirable). Identifying the mechanisms that cause GI to deactivate can facilitate

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efforts to increase GI thermostability, allowing increased reaction temperatures that could substantially improve the economics of HFCS production.

Volkin and Klibanov *(1)* reported that HFCS and a competitive inhibitor to the enzyme, xylitol, greatly stabilized the immobilized form of GI (IGI) from *Streptomyces olivochromogenes* at high temperatures. The authors also found that at 60°C, IGI deactivation was related to

1. Oxidation of the enzyme's cysteine residues.
2. Heat induced reactions with HFCS.
3. Impurities present in the reaction medium.

In a review published by Quax *(2)*, the author cited that substituting an arginine residue for a lysine residue at the subunit interface of *Actinoplanes missouriensis* GI increased the enzyme's thermostability by two- to threefold. Visuri et al. *(3)*, in turn, reported that the crystalline form of GI from *S. rubiginosus* was more stable in the presence of substrate, whereas in buffer solution, the native enzyme (SGI) was more stable. The loss of activity for SGI was directly proportional to protein precipitation. The enzyme first underwent some precipitation. Once precipitation ceased, the inactivation of the enzyme also stopped. They also claimed that SGI deactivation in HFCS was related to Maillard (browning) reactions that took place between the enzyme and the sugar, which resulted in the formation of a sugar–protein complex. The crosslinked form of GI was not susceptible to the Maillard reaction, possibly because the glutaraldehyde crosslinker had reacted mainly with the GI lysine residues, which were the very residues prone to the deleterious Maillard reaction.

Although previous investigations have identified possible mechanisms for GI inactivation, a thorough investigation of the mechanisms for inactivation of GI from *S. rubiginosus* has not been previously conducted. The objective of this study, therefore, is to further establish the contributions of thiol oxidation of the cysteine residue and the Maillard-like reaction to the thermoinactivation of both SGI and IGI from *S. rubiginosus*, one of the most prevalent commercial forms of GI.

A thorough understanding of the underlying mechanisms of thermoinactivation could facilitate efforts to increase the enzyme's thermostability, ultimately improving economics by reducing the need to purchase new enzymes and hence reduce the cost of HFCS production as a whole. One of the most effective ways to achieve stabilization is by the use of immobilized enzyme *(4)*. Multipoint covalent attachment to solid matrices has been used to stabilize several industrial enzymes *(5)*. The formation of the rigid enzyme-support linkage provides substantial kinetic and thermodynamic stabilization of the 3D structure of the active catalytic site. The immobilized enzyme molecules may also be stabilized against denaturing agents that induce enzyme unfolding that can destroy the active site *(6)*.

In this study, GI has been immobilized on silica gel. The contributions of the Maillard-like reaction and thiol oxidation of the cysteine residue

of both SGI and IGI to the thermoinactivation of the enzyme were then determined.

Materials and Methods

Chemicals

SGI (E.C. 5.3.1.5. D-xylose ketol isomerase) from a genetically modified strain of *S. rubiginosus*, was supplied by Genencor (Rochester, NY) as Gensweet SGI. Maleic acid, cobalt chloride, magnesium sulfate, D-glucose, D-fructose, sucrose, D-galactose, bovine serum albumin, calcium nitrate, 2-mercaptoethanol, hydrochloric acid, acetone, sodium phosphate dibasic, sodium phosphate monobasic, and activated carbon, were purchased from Fisher Scientific (Unionville, ON, Canada). D-xylose, D-Mannose, guanidine hydrochloride, 5,5′-dithiobis, Tris (Trizma base), Coomassie Blue G250, 3-aminopropyl-triethoxysilane (APES), citric acid, and sodium citrate tribasic dihydrate were supplied by Sigma (Oakville, ON, Canada). Phosphoric acid (85%) and ethanol (95%) were supplied by VWR Scientific. EDTA was purchased from BioShop (Burlington, ON, Canada). Glutaraldehyde (50% [w/v] in water) was purchased from ACROS (Morris Plains, NJ).

The silica gel, supplied by W. R. Grace & Co (Columbia, MD) was of Type 654: 100 × 200 mesh (75–150 μm), 260–340 m^2/g surface area, and average pore diameter of 183–287 Å. GI activity assays were performed at pH 6.85 in 0.2 maleic acid buffer that contained 0.02 *M* magnesium sulphate and 0.001 *M* cobalt chloride. Both batch and continuous GI thermoinactivation studies were carried out at pH 8.0 in 0.05 *M* Tris-HCl buffer that contained 0.02 *M* magnesium sulphate.

Immobilization of Enzyme

The method of enzyme immobilization was adapted from that suggested by Weetall and Filbert *(7)* and Wiseman *(8)*. For the silanization process, 10% APES in acetone was used. In the support activation step, the APES-treated silica gel was incubated in glutaraldehyde, prepared by purification with activated carbon, then diluted to 4% (w/v) using 0.5 *M* citric acid buffer (pH 4.8). In the enzyme-coupling step, the modified silica gel was incubated in dialyzed SGI (0.1 *M* phosphate buffer [pH 7.2]) for about 24 h at room temperature. The moisture content of the resulting IGI was in the range of 60–70%.

Standard GI Assay

The standard GI activity, defined as the change in fructose concentration over 20 min of reaction at 60°C, was used to quantify the catalytic activity of the enzyme, and to quantify the efficiency of immobilization. In the assay, 4 mL of total of 1 *M* glucose substrate in buffer was incubated in a 7-mL glass vial immersed in the jacketed batch reactor at 60°C. For SGI

assays, the enzyme was diluted by a factor of 191. For IGI assays, about 0.4 g of wet IGI was used. One milliliter of the reaction medium was sampled at time zero and 20 min, respectively, and the reaction was stopped by adding 0.25 mL of 20% HCl to 1 mL of sample. The sample was analyzed for fructose and glucose content using an HPX-87C carbohydrate column (BioRad, Mississauga, ON, Canada) in a Perkin Elmer high-performance liquid chromatograph (HPLC) with a refractive index detector. The activity of the immobilized enzyme was based on its dry weight, which was determined gravimetrically.

GI Activity Assay for Batch Thermoinactivation Study

In this assay, 4 mL of 1 *M* glucose in buffer was incubated in a 7-mL glass vial immersed in the jacketed batch reactor at 60°C. When the reaction medium reached 60°C, 0.5 mL of SGI (already diluted about 1 : 17) from the thermoinactivation experiment was added. For the IGI assay, one milliliter of IGI suspension from the thermoinactivation experiment was pipeted into 4 mL of reaction medium that had been preincubated at 60°C. One mL of the reaction medium was sampled at time zero and 20 min, respectively, and the reaction was stopped by adding 0.25 mL of 20% HCl to 1 mL of sample, which was subsequently analyzed using HPLC. The activity of IGI was normalized with respect to its dry weight, which was determined gravimetrically.

Protein Assay

Total protein concentrations of the enzyme solutions were determined by the Bradford method *(9)*.

Thiol Assay

Ellman's reagent was used to test for thiol content in IGI and SGI. The Ellman's reagent stock contained 97.8% (v/v) 6 *M* guanidine HCl and 1 m*M* EDTA, 1.1% m*M* Ellman's reagent, and 1.1% 1 *M* NaOH. For the IGI thiol assay, a 1 mL suspension of IGI in reaction medium (or 1 mL of supernatant of centrifuged reaction medium, which was used as the blank) was incubated in 2.7 mL of Ellman's reagent stock. For the SGI thiol assay, 100 μL of SGI in reaction medium was added to 0.9 mL of Ellman's reagent stock. The mixture was incubated at room temperature for about 15 min and the absorbance at 412 nm was measured. β-Mercaptoethanol was used as the standard.

Thermoinactivation Study in Batch Reactors

GI thermostability experiments in batch reactors were carried out at 60, 70, and 80°C. The contributions of thiol oxidation and Maillard reaction on the thermoinactivation of GI were studied. All thermoinactivation experiments were carried out in stirred jacketed batch reactors. The contents were mixed using a magnetic stirrer. These experiments lasted from about 20 h (for the 80°C runs) to about 60 h (for the 60°C runs).

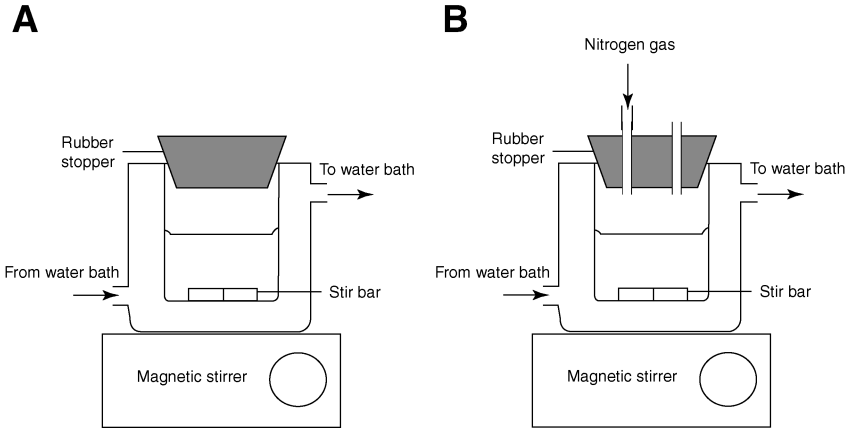

Fig. 1. Schematic of jacketed batch reactors for **(A)** GI thermoinactivation study under air-saturated condition and **(B)** GI thermoinactivation study under reduced oxygen conditions (nitrogen sparged).

Thiol Oxidation Effect

The thiol content of IGI and SGI was determined using the procedures previously described under "Thiol assay." For experiments that required reduced dissolved oxygen content, the reaction medium was sparged with nitrogen for at least 30 min before the experiment was started. Initially, the reaction medium (without enzyme) was sparged by submersing the nitrogen supply below the liquid surface. Once the reaction was initiated (by adding enzyme), the nitrogen supply line was withdrawn and placed in the headspace above the reaction medium to maintain oxygen-depleted conditions. Oxygen levels were measured using a Biological Oxygen Monitor from Yellow Springs Instruments (YSI, Yellow Springs, OH) with a high sensitivity membrane (YSI model 5794). Prereaction sparging with nitrogen was able to reduce the dissolved oxygen content by about 95% compared with air saturation, and this was maintained during reaction by the supply of nitrogen into the headspace. Even though nitrogen sparging could not completely eliminate oxygen from the reaction medium, there was nonetheless a significant reduction in oxygen content, so that the impact of oxidation could be studied. A schematic of the experimental setup is shown in Fig. 1. Evaporation of reaction medium at high temperatures was accounted for by replenishing the medium with nitrogen-sparged deionized water, which was heated to the same reaction temperature as the reaction medium. Samples were collected periodically and tested for thiol content and GI activity.

Maillard-Like Reaction Effect

Different sugars, namely, glucose, sucrose, galactose, and xylose were added to the reaction medium to examine the effect of the Maillard-like

reaction on the thermoinactivation of IGI and SGI. Samples were taken and tested for their GI activity.

Thermoinactivation Study in Continuous Reactors

Continuous glucose isomerization using IGI was carried out in a 1×30 cm^2 jacketed PTFE liquid chromatography column (Sigma) with a bed volume of 24 mL. The reaction medium contained 1 *M* of glucose in 0.05 *M* Tris-HCl buffer (pH 8.0) containing 0.02 *M* $MgSO_4$. The reaction medium was pumped through the reactor at a flow rate of approx 0.4 mL/min using a peristaltic pump. Samples were taken periodically from the outlet of the reactor and analyzed for fructose and glucose using HPLC. To determine the cumulative fructose produced over the entire length of each experiment, the product was collected and tested for the "pooled" glucose and fructose concentrations at the end of the experiment, using HPLC. These experiments lasted between 5 and 15 d, depending on the enzyme loading and temperature. The activity of IGI was determined from the outlet fructose concentration. Once the outlet fructose concentration begins to drop below its equilibrium level, the amount of active enzyme in the reactor is directly proportional to the amount of fructose produced. Beyond this point, the relative activity of the enzyme at time (t) can be determined from a ratio of the instantaneous fructose concentration to the fructose concentration at equilibrium. The first-order deactivation kinetics model (Eq. 2) was used to model IGI deactivation under continuous isomerization conditions, following linear regression of the semilog enzyme activity profiles.

The turnover number (TON) of the enzyme is defined as the ratio of the amount of fructose produced by the enzyme from time zero to time (t), to the amount of enzyme consumed or inactivated over the same interval. To calculate the TON, the cumulative total mass of fructose produced and determined experimentally, was divided by the total mass of the enzyme consumed (i.e., deactivated) during the reaction.

Enzyme Deactivation Model

A first-order enzyme deactivation model was used to represent GI deactivation kinetics. First-order deactivation model is consistent with the disruption of a single bond or "sensitive structure," or the occurrence of a single lethal event or a "single hit" *(10)*. Gibbs et al. *(11)* applied the extended Lumry–Eyring model to describe GI deactivation, where the native enzyme (N) first unfolds reversibly to the unfolded species (U), which is catalytically inactive, and then deactivates irreversibly through first-order kinetics to a deactivated species (D). First-order enzyme deactivation kinetics has also been successfully used to describe the deactivation of GI *(12)*. Treating the native and the unfolded forms, i.e., N and U, as one single catalytically active species, (E), and assuming that the subsequent irreversible enzyme

deactivation is a first-order event, the enzyme deactivation kinetics can be described by:

$$\frac{d[E]}{dt} = -k_d[E] \tag{1}$$

Integrating Eq. 1 from $t = 0$ to t gives:

$$\frac{[E]}{[E_0]} = \exp(-k_d t) \tag{2}$$

Statistical Analysis of Data

Replicates of the kinetics and thermoinactivation studies were produced for most conditions. The HPLC assay was subject to a mean of ±2% and a maximum deviation of ±10%. To account for experimental variability and for statistically justifiable comparisons between experimental runs, 95% confidence intervals (CI) were computed for experiments for the thermoinactivation and kinetics studies. The equation used *(13)* to compute the 95% CI was:

$$95\%\,\mathrm{CI} = t_{\alpha/2} \times s_e / (S_{xx})^{0.5} \tag{3}$$

where $t_{\alpha/2}$, studentized test statistic; α, significance level (5%); s_e, standard error; and $S_{xx} = \sum x^2 - (\sum x)^2/(\text{number of data points})$.

Initial comparisons between experimental runs were based on 95% CI, to establish if the results were statistically different. If a difference was confirmed, the degree of difference was then determined using the extreme values of the CIs, i.e., the "worst-case scenario" for each run, based on a 95% confidence level. For example, with A (10 ± 1) and B (5 ± 2), it is apparent that A is statistically different from B, based on a 95% confidence level. In the worst-case scenario, A is at least approx 1.3 times greater than B, calculated using the CIs as follows: $(10-1)/(5+2) = 9/7$ approx 1.3. This approach may not quantify the actual magnitude of the difference between two means, but it serves as a more stringent test of the degree of difference between trial conditions. In comparisons where such an approach has been used, the magnitude of the difference will be stated, preceded by the clause "at least," for example, "at least" 1.3 times greater than B, as written earlier.

Results and Discussion

GI Immobilization

GI from *S. rubiginosus* was successfully immobilized on silica gel through covalent immobilization with support silanization. The activity yield of the immobilization process was between 20 and 40%, and the IGI

Table 1
Effect of Nitrogen Sparging and Temperature on Thiol Content of IGI and SGI Under Various Conditions in HFCS

		Rate of decrease, m, h^{-1} (no. of data points)	
		60°C	80°C
IGI	N_2 sparging	0.007 ± 0.003 (18)	0.037 ± 0.010 (19)
	No N_2 sparging	0.014 ± 0.002 (20)	0.057 ± 0.014 (19)
SGI	N_2 sparging	0.008 ± 0.008 (2)	0.010 ± 0.009 (19)
	No N_2 sparging	0.005 ± 0.007 (20)	0.054 ± 0.015 (19)

Values of *m* shown as mean ± 95% CI, based on three replicates.

Table 2
First-Order Deactivation Constants and Their Corresponding Half-Lives for IGI and SGI at Various Temperatures in HFCS

		First-order k_d/h^{-1} (half-life [h])	
		60°C	80°C
IGI	N_2 sparging	0.008 ± 0.003 (89)	0.042 ± 0.024 (16)
	No N_2 sparging	0.025 ± 0.005 (27)	0.060 ± 0.018 (12)
SGI	N_2 sparging	0.002 ± 0.002 (286)	0.038 ± 0.012 (18)
	No N_2 sparging	0.005 ± 0.002 (145)	0.133 ± 0.030 (5)

Values of k_d shown as mean ± 95% CIs, based on three replicates.

was retained on the support during production of HFCS. The IGI produced had an activity of 3.1 ± 0.5 unit GI/g (mean ± standard deviation).

Contribution of Thiol Oxidation to GI Thermoinactivation

The contribution of thiol oxidation of the cysteine residue to the thermoinactivation of SGI and IGI at 60 and 80°C in HFCS, which initially contained only 1 *M* glucose, was investigated. The terms "nitrogen-sparged" or "nitrogen-sparging" are used to denote conditions where the oxygen content of the reaction medium was reduced by approx 95% compared with air-saturated conditions.

Table 1 shows the rate of thiol oxidation (m, in h^{-1}), for SGI and IGI under various conditions. The first-order deactivation kinetics model (Eq. 2) was used to describe the enzyme deactivation because of thiol oxidation, and linear regression was performed on the enzyme activity profiles obtained for IGI and SGI at different temperatures in HFCS, which initially contained only 1 *M* glucose. The best-fit first-order deactivation constants, k_d, and their corresponding half-lives are listed in Table 2. Figures 2 and 3 show the stabilities of SGI and IGI under air-saturated and nitrogen-sparged conditions at 60 and 80°C, respectively.

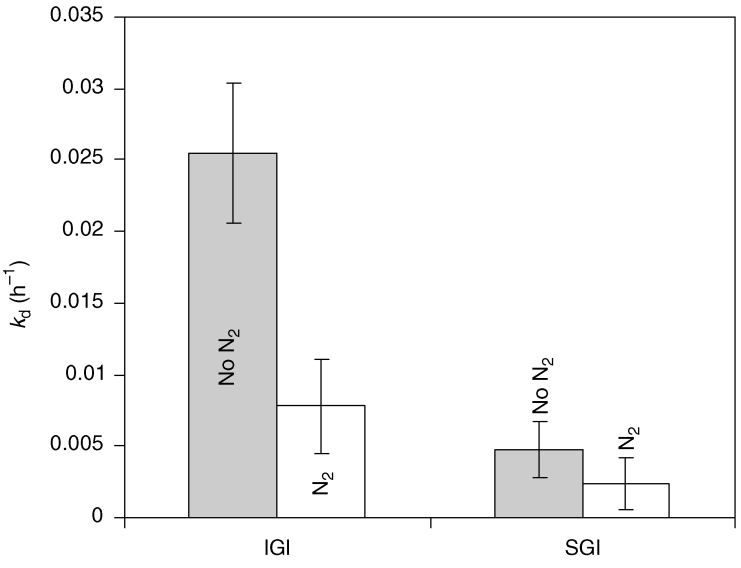

Fig. 2. Deactivation constant (k_d), for IGI and SGI at 60°C in batch reactors. Data shown as mean ± 95% CI. Enzymes were incubated in Tris-HCl buffer (pH 8.0) that initially contained 1 *M* of glucose.

In general, the rate of thiol oxidation was higher when the enzyme was incubated under air saturation. For SGI at 60°C, experimental data showed a negligible rate of thiol oxidation, based on a 95% CI. There was also no significant difference in the extent of oxidation of the thiol group between the nitrogen-sparged and air-saturated batches, which suggests that the cysteine residue in SGI is not susceptible to oxidation at 60°C. For IGI at 80°C, there was no significant difference between the slopes representing the rates of decrease of thiol content under air and nitrogen saturation. It can also be noted that at 80°C, under air saturation, there was no difference between the rate of thiol oxidation for IGI and SGI. On the other hand, with nitrogen sparging, the rate of thiol oxidation for IGI was *at least* 1.4 times higher than that of SGI at 80°C (0.027/0.019). It was also visually observed that for SGI, protein precipitation became more and more severe at higher temperatures. At 80°C, SGI precipitation occurred within the first 30 min of reaction. Reaction medium browning, measured spectrophotometrically at 330 nm, also became more noticeable for SGI as the temperature increased from 60 to 80°C.

At 60°C without nitrogen sparging, SGI was more stable than IGI. Using nitrogen sparging to reduce the dissolved oxygen content enhanced IGI stability by *at least* 1.8 times (0.020/0.011). Conversely, at a 95% confidence level, no difference was observed between the nitrogen-sparged and air saturation runs for SGI. The thiol concentration profile of IGI at 60°C shows that thiol oxidation occurred in parallel with IGI deactivation. This suggests that thiol oxidation contributed to the thermoinactivation of IGI.

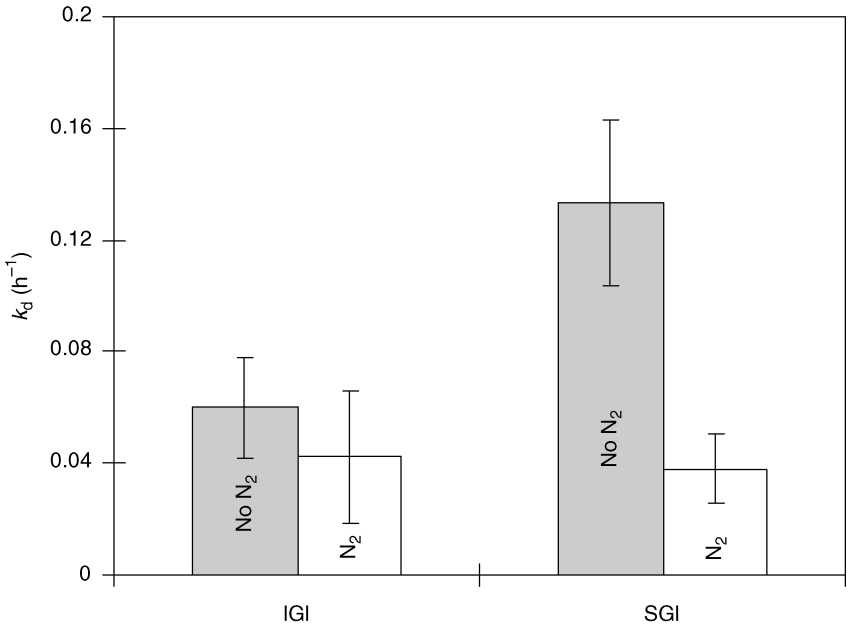

Fig. 3. Deactivation constant (k_d), for IGI and SGI at 80°C in batch reactors. Data shown as mean ± 95% CIs. Enzymes were incubated in Tris-HCl buffer (pH 8.0) that initially contained 1 *M* of glucose.

No conclusion can be drawn regarding the relation between thiol oxidation and deactivation for SGI because there was little (if any) change in the thiol content of SGI at 60°C, irrespective of the oxygen content in the system.

Nitrogen sparging of SGI at 80°C reduced the extent of thiol oxidation in SGI, and at least doubled the stability of the enzyme compared with that under oxygen saturation. However, for IGI there was no statistically significant difference in half-life between the nitrogen-sparged vs air-saturation conditions. Nitrogen sparging did not reduce the rate of thiol oxidation of the cysteine residue of IGI (Table 1). Therefore, there was not enough evidence to suggest that thiol oxidation was a cause of IGI deactivation, unlike at 60°C. The key conclusion that can be drawn from these observations is that thiol oxidation is a likely reason for the loss of activity of SGI at 80°C.

At 80°C, under air-saturation conditions, IGI was *at least* 1.3 times (0.103/0.078) more stable than SGI, even though there was no statistically significant difference in the rate of thiol oxidation for both enzyme forms. When nitrogen sparging was used, there was no statistically significant difference between the stability of IGI and SGI, in spite of the observation that the rate of thiol oxidation for IGI was *at least* 1.4 times higher than that of SGI. There was also no statistically significant difference between the half-lives of IGI (under air saturation) and SGI (under nitrogen-sparged conditions). These observations indicate that, compared with SGI, the stability of IGI was less sensitive to thiol oxidation. This is also an indication

Table 3
Relative Change in Thiol Oxidation Rate and Half-life for IGI and SGI When Reaction Temperature was Increased From 60 to 80°C

		Temperature increased from 60 to 80°C	
		Relative increase in thiol oxidation rate	Relative decrease in half-life
IGI	N_2 sparging	2.7	1.6
	No N_2 sparging	2.7	1.4
SGI	N_2 sparging	1.0	6.5
	No N_2 sparging	3.3	15

that immobilization protected the enzyme against inactivation owing to thiol oxidation.

The relative increases in the rate of thiol oxidation and the half-life of IGI and SGI when the reaction temperature was increased from 60 to 80°C are shown in Table 3. Based on Table 3, it can be concluded that nitrogen sparging did not reduce the extent of thiol oxidation of IGI. Immobilization of GI probably affected the structure of the enzyme so that the increase in temperature resulted in equal increase in the thiol oxidation rate of the enzyme, regardless of the oxygen content in the reaction medium. Nitrogen sparging did not affect the rate of thiol oxidation in SGI when the temperature was increased from 60 to 80°C; however, the stability of the enzyme still decreased by *at least* a factor of 6.5. On the other hand, under air saturation, the same increase in temperature resulted in *at least* a 15-fold decrease in stability. This suggests that the change in stability was not owing to thiol oxidation alone, even though thiol oxidation was important. Clearly, other processes also contributed to the loss of SGI activity.

Volkin and Klibanov *(1)* reported that the four monomeric subunits of *S. olivochromogenes* GI molecule each contain one cysteine residue imbedded in the interior of the hydrophobic core of the enzyme. At 60°C, it was possible that SGI did not undergo significant conformational change, and the cysteine residue remained protected in the hydrophobic core of the enzyme molecule. Conversely, at 80°C, SGI experienced significant conformational changes, as implied by the severe enzyme precipitation that occurred within the first 30 min of the reaction; these conformational changes exposed the enzyme's cysteine residues to oxygen in the reaction medium. Conversely, when the temperature increased from 60 to 80°C, IGI did not undergo enzyme precipitation, suggesting that IGI did not undergo severe conformational changes, unlike SGI. These observations suggest a relationship between the enzyme's conformational change and the adverse effects of thiol oxidation.

Immobilization made the enzyme more susceptible to thiol oxidation at 60°C, perhaps by a structural change in the enzyme during the immobilization

process that caused the enzyme's cysteine residue to be more readily accessible for oxidation. Such a structural change in the enzyme after immobilization may have also suppressed the destabilizing effect of thiol oxidation on the enzyme at 80°C.

Contribution of the Maillard-Like Reaction to GI Thermoinactivation

Independent reaction kinetics studies by Lim *(14)* on both soluble and immobilized GI from *S. rubiginosus* have shown a reduction in total sugar (glucose and fructose) in the reaction medium that paralleled the loss of the enzyme activity. This is a strong indication of the occurrence of the Maillard-like reaction between the enzyme and the sugars. The effect of different sugars on the deactivation of GI was investigated at 60, 70, and 80°C by incubating the enzyme in 1 mol/L of either sucrose, xylose, or galactose, followed by a subsequent activity assay. Sucrose, a nonreducing disaccharide is the least reactive with proteins, whereas xylose has the greatest reactivity *(3)*.

The first-order deactivation kinetics model (Eq. 2) was used to model GI deactivation. Linear regression was performed on the semilog enzyme activity profiles. The best-fit first-order deactivation constants and their corresponding half-lives are listed in Table 4. These results are based on a single trial with each sugar; each trial contained six data points. The use of data from only a single run for each condition might not reflect the overall variability that would otherwise be observed from experimental replicates under the same condition. However, all the experiments in this study were performed simultaneously, to reduce variability owing to enzyme activity and other biological artifacts. Therefore, it is still reasonable to compare the experimental data within the study.

Experimental trials with xylose showed greater variability/fluctuation, for both IGI and SGI. The fluctuations were also manifested in relatively larger standard deviations of GI stability in xylose (Table 4). This could be because of the fact that xylose, a compound with greater affinity for GI than glucose (the substrate in the activity assay) was bound to the enzyme's active site during incubation. This also accounts for the observation that GI preincubated in xylose had initial activities (i.e., at time zero of incubation) that were 80–87% lower than those for GI preincubated in sucrose and galactose. These relatively low activities ultimately introduced greater variation in the data arising from experiments with xylose.

The stabilities of SGI and IGI depend both on the presence of the sugars and reaction temperature. At 60°C, SGI was *at least* 1.2 times (0.019/0.016) more stable in galactose than in xylose, whereas at 70 and 80°C, there was no statistically significant difference between the stability of SGI in either galactose or xylose. However, at both 60 and 80°C, the stability of SGI incubated in sucrose was dramatically more than that observed when the enzyme was incubated in galactose or xylose. At 80°C, SGI was about three to five times more stable in sucrose than in galactose or xylose, respectively. At 60°C, there was no statistically significant difference in

Table 4
Effect of Exogenous Sugars on the Deactivation of GI

Sugar used for incubation	Enzyme form	First-order k_d, h^{-1} (half-life, h)		
		60°C	70°C	80°C
1 *M* sucrose (least reactive with proteins)	IGI	0.020 ± 0.002 (35.0)	No data available	0.121 ± 0.061 (5.7)
	SGI	Very stable k_d approx 0	No data available	0.025 ± 0.004 (28.0)
1 *M* galactose	IGI	0.023 ± 0.012 (30.1)	0.051 ± 0.026 (13.5)	0.118 ± 0.054 (5.9)
	SGI	0.014 ± 0.002 (49.5)	0.088 ± 0.003 (7.8)	0.172 ± 0.063 (4.0)
1 *M* xylose (most reactive with proteins)	IGI	0.028 ± 0.021 (24.4)	0.138 ± 0.056 (5.0)	0.243 ± 0.040 (2.8)
	SGI	0.073 ± 0.054 (9.5)	0.104 ± 0.059 (6.7)	0.308 ± 0.114 (2.2)

k_d values are shown as mean ± 95% CIs.

stability of IGI incubated in the three different sugars. However, at 70°C, IGI was *at least* 1.1 times more stable in galactose than in xylose, whereas at 80°C, IGI was *at least* 1.1 times more stable in sucrose than in xylose, and *at least* 1.2 times more stable in galactose than xylose. Browning of the reaction medium was also visually observed and was more prevalent at higher temperatures. Browning was most prominent in sucrose, and least prominent in xylose.

Based on changes in enzyme half-life on incubation in different sugars at any particular temperature, it is apparent that immobilization suppressed the effects of the Maillard-like reaction on the thermoinactivation of GI, especially at lower temperatures (<80°C). Such suppression could be because of the glutaraldehyde crosslinker used to activate the silanized support before the enzyme-coupling step. It is expected that glutaraldehyde reacted mainly with the lysine residues on the GI, the very residues most susceptible to the destructive Maillard reaction *(3)*. The Maillard-like reaction between the enzyme's lysine residue and the sugars was less prominent in IGI, possibly because the lysine residues had been used to link the enzyme to the support, and hence, was not available for reaction with the sugars. Even though definitive detection of the Maillard reaction between the enzyme and the sugars was not established in this study, experimental observations of the stability of SGI and IGI under sugars of different reactivities, and the definitive loss of total sugar in the reaction medium that occurred concurrently with the loss of enzyme activity *(14)* are consistent with a Maillard-like reaction between the enzyme and the

Table 5
Comparison of TONs (Gram Fructose Produced/Gram Enzyme Consumed) and Deactivation Rate Constants (Mean Value ± 95% CI) for IGI Under Continuous Isomerization Conditions

		60°C	80°C
IGI	TON, per g wet mass	72	78
	TON, per g dry mass	2.4×10^2	2.6×10^2
	k_d, h^{-1} (half-life [h])	0.0085 ± 0.0022 (82)	0.019 ± 0.005 (36)

sugars. Furthermore, the results from these trials are consistent with those of Quax *(2)*, who previously suggested that the Maillard reaction between a lysine residue and HFCS was responsible for the thermoinactivation of SGI from *A. missouriensis*.

GI Thermoinactivation in Continuous Reactors

Thermoinactivation studies of the IGIs in continuous reactors provided thermostability information under conditions that more closely reflect actual industrial operation. Batch operation allows inhibitors to accumulate, whereas continuous operation carries out these inhibitors with the effluent, reducing their impact on the enzyme. Therefore, continuous reactors provided another platform to investigate the effect of the Maillard reaction on the thermoinactivation of IGI.

A sample calculation of TON is given as follows: at 60°C, an average of 12.8 g of fructose was produced after 265 h of continuous reaction catalyzed by 2.0 g of IGI, based on two experimental replicates. The fraction of active IGI left at the 265th h was 0.11, calculated using the enzyme deactivation model with the best-fit k_d value. Thus, the total mass of wet IGI consumed was 1.8 g. Therefore, the TON was 7.2 g fructose/g wet IGI consumed, and the TON per gram dry mass was 239. The best-fit first-order deactivation constants and the TONs for all experimental conditions are shown in Table 5.

During continuous isomerization at 60°C, IGI was *at least* 1.9 times more stable than IGI under batch isomerization conditions, based on the computed k_d values. At 80°C, IGI during continuous isomerization was *at least* 1.8 times more stable than it was during batch processing, a similar improvement to that observed at 60°C.

The difference in the relative stability of IGI in batch and in continuous reaction systems might be because of differences in the extent of the Maillard-like reaction in the two systems. During batch processing, equilibrium was reached within 10–20 min, and the enzymes were then continuously exposed to high concentrations of sugars for an extended period of time. During this period, samples were removed and subjected to a kinetics assay for enzyme

Table 6
Factors Contributing to Thermoinactivation of IGI and SGI

	Factors causing deactivation		Relative stability (in HFCS, under air-saturation conditions)
	SGI	IGI	
60°C	Maillard-like reaction	Thiol oxidation and Maillard-like reaction (relatively mild)	SGI > IGI
80°C	Thiol oxidation and Maillard-like reaction	Maillard-like reaction (relatively mild)	IGI > SGI

activity. By comparison, in the continuous systems, certain regions of the bed were exposed to lower concentrations of fructose, and the loss of activity was directly determined from the change in outlet fructose concentrations over time. Consequently, the exposure of the enzymes to sugars responsible for the Maillard-like reaction was different, with higher levels of exposure in the batch system. Furthermore, in the batch system, these sugars had a longer residence time in the reactor and hence, more time for the Maillard-like reaction to proceed. The resulting accumulation of byproducts in the batch system may have enhanced the inactivation of the enzyme under batch conditions. In the continuous reactors, the sugars were constantly removed from the system, which therefore limited the amount of byproduct formation as a result of the Maillard-like reaction. The overall effects of thiol oxidation and the Maillard-like reaction on the stability of SGI and IGI are summarized in Table 6.

Conclusions

S. rubiginosus GI was successfully immobilized onto silica gel using a covalent-binding method following derivitization with APES. The immobilized enzyme had high enzyme retention on the support during glucose isomerization reaction. For SGI, the Maillard-like reaction was the main contributor to inactivation at 60°C, whereas at 80°C, both the Maillard-like reaction and thiol oxidation were significant. For IGI, a mild effect of the Maillard-like reaction was observed at 60°C and 80°C. Thiol oxidation was significant at 60°C, but not at 80°C. Furthermore, SGI was more stable than IGI at 60°C, but the converse was true at 80°C. Therefore, immobilization shifted the thermoinactivation mechanism of GI. At 80°C, there was evidence that thiol oxidation of IGI still occurred, but its impact on IGI deactivation was reduced. Thermoinactivation of IGI at temperatures higher than 60°C could be also caused by deamidation of the asparagine and/or glutamine residues of the enzyme *(1)*.

Nomenclature

$[E]$	active enzyme concentration (M)
$[E_0]$	initial enzyme concentration (M)
k_d	degradation rate constant in Eq. 2 (h^{-1})
IGI	immobilized GI produced at the lab
SGI	soluble GI supplied by Genencor
t	time (h)

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